Characterization and Practical Benefits of Keratinocytes Cultured in Strontium-Containing Serum-Free Medium

Fukumi Furukawa, M.D., J. Clark Huff, M.D., Martin B. Lyons, B.A., William L. Weston, M.D., and David A. Norris, M.D.

Department of Dermatology, University of Colorado School of Medicine, Denver, Colorado, U.S.A.

Strontium (Sr^{2+}) can substitute for Ca^{2+} and stimulate a variety of functions of numerous types of cells. The purpose of this study was to investigate the details of the biologic effects of Sr^{2+} on human keratinocyte growth, cell cycle, viability, and differentiation and to compare these effects with Sr^{2+} effects on cultured skin melanocytes. Cultured keratinocytes stimulated with 1.0–3.0 mM Sr^{2+} showed higher viability and almost a twofold increase in cell number compared with those grown in a standard calcium concentration. Time course studies revealed that 2.0 mM Sr^{2+} had no effects on growth of cultured melanocytes or fibroblasts. Sr^{2+} increased the percentage of cultured keratinocytes in G_2/M phase, with a decrease in cells in G_0/G_1 phase. This effect of Sr^{2+} on the cell cycle was not seen in cultured melanocytes or fibroblasts. A 2 mM concentration of Sr^{2+} produced an increase in low-density keratinocytes separated by a Percoll gradient. In addition, increased expression of human fibronectin was observed in the cytoplasm and on cell membranes of keratinocytes cultured in Sr^{2+}. Sr^{2+} can be of practical benefit in the culture of human keratinocytes in serum-free medium, increasing the viability and proliferative rate and producing a more uniform population of basaloid cells with increased expression of cell surface fibronectin. *(J Invest Dermatol 90:690–696, 1988)*

Growth of keratinocytes in tissue culture is determined by the viability and proliferative capability of the cells, the seeding density, the surface conditions in culture dishes or flasks, and the components in the culture medium. High seeding densities enable serial culture of keratinocytes obtained from adult skin as well as from neonatal foreskin [1–3]. Human keratinocytes (HKs) have been successfully cultivated on lethally irradiated 3T3 cell feeder layers [1], on collagen- and/or human fibronectin (HFN)-coated surfaces [3–5], and on natural extracellular matrix-coated flasks [6].

Supplemental factors in culture medium are of critical value in the successful culture of keratinocytes. Epidermal growth factor [7] and serum-derived hormones regulate mammalian cell growth in vitro [8]. Keratinocyte growth depends in large part on endocrine stimulation [9]. In addition, cell-conditioned medium, fetal calf serum (FCS), organ extracts, essential nutrients, pH, and calcium (Ca^{2+}) play essential roles in keratinocyte cultures [10–13].

It is well known that the presence of Ca^{2+} in the culture medium induces growth, proliferation, and differentiation of cultured keratinocytes [13]. In many secretory processes, strontium (Sr^{2+}) can substitute for Ca^{2+} [14,15]. A recent report showed that Sr^{2+} has mitogenic effects on growth of cultured keratinocytes and Sr^{2+} can substitute for Ca^{2+} in stimulating keratinocyte proliferation but not terminal differentiation [16].

We examined Sr^{2+} effects on human keratinocyte growth, cell cycle, viability, and differentiation and compared them with strontium effects on human skin melanocytes and fibroblasts.

MATERIALS AND METHODS

Culture Medium We selected optimal culture media for each of the three cell types studied. The basic medium for human keratinocytes (basic HK medium) used in the present study consists of MCDB153 (Lot no. 906661004, 6020202 Irvine Scientific, Irvine, CA) supplemented with 10 ng/ml epidermal growth factor (Collaborative Reserach, Boston, MA), 5 μg/ml insulin (Sigma, St. Louis, MO), 1.4 μM hydrocortisone (Sigma), 0.1 mM ethanolamine (Sigma), and 0.1 nM phosphoethanolamine (Sigma). In primary culture and first-passage cell culture, basic HK medium was supplemented with 0.1 mM Ca^{2+} and whole bovine pituitary extract (wBPE,60 μg/ml, Collaborative Research) according to the report of Boyce and Ham [13].

Manuscript received August 4, 1987; accepted November 13, 1987.

This work was supported by Grants AM26427, GM 35068, and AI 16637 from the National Institutes of Health.

F. Furukawa is the recipient of an Upjohn Fellowship from the Dermatology Foundation. His present address is Department of Dermatology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan.

This work was presented at the National SID meeting in 1987 and at the 5th International Meeting on the Differentiation of Normal and Neoplastic Cells in 1987.

Reprint requests to: David A. Norris, Department of Dermatology, B-153, University of Colorado School of Medicine, 4200 E 9th Ave, Denver, CO 80262.

Abbreviations:
AO: acridine orange
EB: ethidium bromide
FCS: fetal calf serum
HEM: human epidermal melanocyte
HFN: human fibronectin
HK: human keratinocyte
IF: immunofluorescence
MoAb: monoclonal antibody
PBS: phosphate-buffered saline
PI: propidium iodide
PMA: phorbol 12-myristate 13-acetate
PPD: paraphenylenediamine
RT: room temperature
WBPE: whole bovine pituitary extract

0022-202X/88/$03.50

For the culture of melanocytes, MCDB153 was supplemented with 2.5 nM cholera toxin (Sigma), 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma), 5% fetal calf serum (FCS, Irvine Research), and 2.0 mM Ca^{2+}. This culture medium was termed human epidermal melanocyte medium (HEM medium).

The baseline Ca^{2+} concentration of MCDB153 used in most experiments was 0.03 mM. Ca^{2+}-free MCDB153 medium was obtained from Clonetics Corporation (San Diego, CA). Strontium chloride hexahydrate ($SrCl_2 \cdot 6H_2O$) and calcium chloride ($CaCl_2$) were purchased from Aldrich Chemical Company (Milwaukee, WI) and Sigma, respectively.

Cell Culture Keratinocytes and melanocytes for cell culture were derived from neonatal foreskin obtained from circumcisions. The subcutaneous fat tissue was trimmed from the foreskins; then the skin was cut into 2 × 2-mm pieces. The skin specimens were incubated in solution A (a Ca^{2+}- and Mg^{2+}-free HEPES-buffered saline, pH 7.6) containing trypsin (2.5 mg/ml, Sigma) at 37°C for 120 min. After epidermal sheets were peeled off the dermis, the effect of trypsin was blocked by adding FCS. The epidermis was agitated gently, centrifuged, and suspended in basic HK medium with 0.1 mM Ca^{2+}, or in HEM medium, which was prepared for cultures of keratinocytes or melanocytes. Protocols for the culture of keratinocytes and melanocytes were those of Boyce and Ham [13] and Eisinger [10], respectively. Primary cultures were expanded in first passage, and then trypsinized into suspension and frozen in aliquots.

For the culture of fibroblasts, separated dermis was incubated in 0.25% collagenase (Sigma) in MCDB153 medium at 37°C for 2 h, and collagenase activity was blocked by adding collagen (Vitrogen, Flow Laboratories, Mclean, VA). The dermis was agitated vigorously, centrifuged at 1,500 rpm (Beckman, TJ-6) for 15 min, and suspended in M199 medium containing 10% FCS. Primary cultures were expanded in first passage and frozen in aliquots.

In the present studies, second-passage cells of each cell type were used and cell purity of each culture was verified by the staining pattern with antikeratin, anti-S100, and antivimentin antibodies. Thus, the effects of various concentrations of Ca^{2+} and Sr^{2+} were tested on highly viable cells first grown in optimal calcium concentrations for primary culture and first passage.

Cell Viability Cell viability was determined by the trypan blue dye exclusion test and acridine orange/ethidium bromide (AO/EB) staining method [17]. For the trypan blue dye exclusion test, cultured cells on dishes were harvested in trypsin (0.05%) and EDTA (0.01%) solution, pipetted into 15-ml culture tubes (Falcon, Oxnard, CA), and placed in medium containing 20% FCS. After centrifugation, cell pellets were resuspended in 0.5 ml of phosphate-buffered saline (PBS). Cell suspensions (100 μl) were mixed with 100 μl of 0.16% trypan blue saline solution and viability was determined in a Neubauer-type hemocytometer. All viabilities were determined in duplicate.

Determination of viability with AO/EB was carried out according to the method of Parks et al [18]. Second-passage cells grown on Lab-Tek chamber slides (Miles, Naperville, IL) were tested. Culture media were aspirated from the chambers and 0.5 ml of AO/EB solution (1 μg AO and 1 μg EB in 1 ml PBS) was added. After 40 s incubation, AO/EB solution was removed, and viability was determined using an epifluorescent microscope (Olympus, Tokyo, Japan). Each test was run in duplicate.

Cell Cycle Analysis Cell cycle analysis of keratinocytes and melanocytes were performed by flow cytometry according to the modified method of Crissman and associates [19,20] and Krishan [21]. Second-passage cells were cultured on T-25 flasks (Corning Glass Works, Corning, NY) for 7 to 10 days. At confluency, cells were harvested in 0.05% trypsin and 0.01% EDTA solution, placed in solution A containing 20% FCS, and then centrifuged. Cell pellets were washed twice with PBS. One million cells per milliter were placed in a test tube. After the final wash, they were resuspended in 1.5 ml cold PBS and 3.5 ml absolute ethanol. Cells were incubated for fixation in this 70% ethanol for 30 min at 4°C. Then cells were washed with PBS, centrifuged, and resuspended in 4.5 ml cold PBS. As a next step, 0.5 ml RNase (10 μg/ml, Sigma) was added, and incubated at 37°C for 30 min. After washing, 5 ml of 6.9×10^{-5} M propidium iodide (PI, Sigma) in 3.8×10^{-2} M sodium citrate was added and incubated for 30 min at room temperature (RT). After they were washed with PBS, cells were filtrated through a 74-μm filter and prepared for cell sorter analysis (Coulter EPICS multiparameter sensor system, Coulter, Hialeah, FL). In this instrument, single cells from a suspension are exposed to an argon ion laser beam (488 nm) and the resulting electrical pulses are stored in the memory unit of a pulse height analyzer and displayed as a histogram. The horizontal axis is divided into 250 channels of increasing linear value, while the vertical axis shows the number of cells recorded in each channel. By this method, the cell ratio of $G_0 + G_1$, S, and $G_2 + M$ phase could be calculated with the microcomputer (Coulter Easy 88 Computer System, Coulter). The theoretical basis for these measurements was reported by Dean and Jett [22] and Gray [23].

Cell Density Cell density was examined by centrifugation through Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), a colloidal silica gradient, according to the methods of Fischer et al [24] and Simon and Green [25] with a slight modification. Second-passage keratinocytes were cultured in 60-mm culture dishes in the basic HK medium with Ca^{2+} or Sr^{2+}. After 7 to 10 days, the cells were harvested by trypsinization. Both the attached and desquamated cells were spun down by low-speed centrifugation [26]. Of the cells thus obtained, 5×10^6 cells in 1.5 ml 30/50% Percoll were mixed with 8.5 ml of 30/50% Percoll in PBS and placed in a 15-ml screw-cap polycarbonate tube. Centrifugation was carried out for 25 min at 800g. Gradients with density-marker beads (Pharmacia) were run simultaneously with the sample gradients to verify density level. The resulting bands of cells were removed carefully with a Pasteur pipette and washed with PBS prior to counting. Cell counting was performed with a Neubauer-type hemocytometer.

Immunofluorescence Studies Second-passage cells grown for 6 to 7 days in Lab-Tek chamber slides were analyzed for immunofluorescence (IF) studies. Cells were washed with PBS for 15 min, rinsed in distilled water for 5 min, and fixed in acetone for 5 min at RT. After they were dried, specimens were incubated with the diluted first antibodies for 60 min at 4°C in a five-slide polypropylene slide mailer [27]. After being washed with PBS, slides were incubated in the diluted second antibodies for 30 min at RT. After being washed with PBS for 15 min, specimens were counterstained with optimally diluted PI solution [28] for 1 min at RT. Specimens were washed in distilled water and in 100% acetone. After drying, one drop of mounting buffer containing paraphenylenediamine (PPD, Fischer Scientific Co., Fairlawn, NJ) according to the method of Huff et al [29] was placed over the stained specimens before application of coverslips. Stained specimens were observed by epifluorescence microscopy. At least 500 cells were counted under ×400 magnification for each antibody tested. In the present studies, the following antisera were used: rabbit antiserum to human keratin (1:100 in dilution, DAKO, Santa Barbara, CA), FITC-labeled goat anti-human fibronectin (1:50 in dilution, Cappel Worthington Biochemicals, Malvern, PA), rabbit antiserum to S-100 protein (1:100 in dilution, Ortho Pharmaceuticals, Raritan, NJ), monoclonal antibody (MoAb) to vimentin (1:10 in dilution, DAKO) and to human skin basal cells (1:10 in dilution, kindly provided by Professor S. Imamura, Kyoto University, Japan) [30], fresh sera from patients with pemphigus vulgaris and bullous pemphigoid (1:20 in dilution), FITC-conjugated goal anti-rabbit IgG (F/P ratio 3.8, 1:50 in dilution, Miles), FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (F/P ratio 3.1, 1:100 in dilution, Tago, Burlingame, CA), and FITC-conjugated rabbit $F(ab')_2$ anti-human IgG (F/P ratio 2.3, 1:100 in dilution, DAKO).

Flow Cytometry Analysis Cell surface antigens were measured in a quantitative fashion by flow cytometry. Methods for cell culture and harvesting were as previously described. After the final

wash with PBS, 1×10^6 cells were resuspended in cold PBS containing 1% bovine serum albumin at 4°C for 2 h. Then, keratinocytes were incubated with the first antibodies at 4°C for 4 h and, if necessary, with second antibodies labeled with FITC at 4°C for 4 h. Antisera examined were anti-keratin, anti-basal cell, anti-HFN, and sera from pemphigus and bullous pemphigoid patients. Stained cells were washed twice with PBS and supernatants were carefully removed; then 100 μl of cold 2% paraformaldehyde solution was added to each tube and mixed thoroughly at once [31]. Finally, these cells were analyzed with a Coulter EPICS multiparameter sensor system by the method of Haftek et al [32].

[^{3}H]Thymidine Incorporation Triplicate wells of keratinocytes (5×10^4/well) were incubated on 96-well culture plates (Falcon) in the base HK medium with 2.0 mM Sr^{2+} or 0.1 mM Ca^{2+} for 4 or more days. Cultures were pulse-labeled with 1 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol, ICN Radiochemicals, Irvine, Ca) for 18 h. Cells were detached in 0.05% trypsin–0.01% EDTA solution at 37°C for 10 min and FCS was added into each well (20% FCS in final volume). The ^{3}H incorporated into trichloroacetic acid-soluble material was measured by liquid scintillation counting.

Statistical Analysis. Students *t*-test and the *t*-test for paired samples were used for statistical analysis. Probability values less than 0.05 were considered significant.

RESULTS

Effects of Sr^{2+} on Viability and Growth of Cultured Keratinocytes. Figure 1 shows the cell viability of keratinocytes on day 7 in culture at different concentrations of Sr^{2+} (0.03–3.0 mM). Cells were cultured in 0.03 mM Ca^{2+} HK medium (base HK medium) and Ca^{2+}-free HK medium. Each point is the mean of three different experiments with duplicate determinations by the AO/EB staining method. At the lower concentrations of Sr^{2+} (0.03, 0.1, and 0.3 mM), cell viability was about 50 to 60%, but at higher concentrations, over 90% of the cells were viable. No significant difference was found between base HK medium (0.03 mM Ca^{2+}) and Ca^{2+}-free HK medium. At both low Ca^{2+} and low Sr^{2+} concentrations, cell viability was poor.

Table I shows the number of viable cells cultured on day 7 in base HK medium (0.03 mM Ca^{2+}) and Ca^{2+}-free HK medium containing different concentration of Sr^{2+} (0.3–3.0 mM). In either Ca^{2+}-free or low Ca^{2+} medium, Sr^{2+} (1.0–2.0 mM) increased the cell number significantly to similar levels. Thus, the effect of Sr^{2+} does not require the presence of Ca^{2+}; however, Ca^{2+} at low levels may augment the maximal Sr^{2+} effect (Table I, [Sr^{2+}]–2.0 mM). Figure 2 shows the relative increase in the number of viable keratinocytes cultured in base HK medium (0.03 mM Ca^{2+}) containing different concentrations of Sr^{2+} (0.3–3.0 mM). The number of cells in base HK medium with 0.1 mM Ca^{2+} and without Sr^{2+} was used as the standard of 100%. Significant elevation of cell number was seen with Sr^{2+} at 1.0, 2.0, and 3.0 mM, with maximal increase at 2.0 mM Sr^{2+}. The number of cells in base HK medium containing only 0.03 mM Ca^{2+} was less than 50% of that in base HK medium with 0.1 mM Ca^{2+} (data not shown). On the basis of these findings, 2.0 mM Sr^{2+} was used as the supplemental trace element in subsequent studies.

In addition, 2.0 mM Sr^{2+} HK medium containing 0.03 mM Ca^{2+} had protective effects on the keratinocytes cultured in Sr^{2+} and then harvested in 0.05% trypsin and 0.01% EDTA solution. Table II shows the comparative viability of cultured keratinocytes grown in 2.0 mM Sr^{2+} HK medium and those grown in basal 0.1 mM Ca^{2+} HK medium. In the 2.0 mM Sr^{2+} HK medium, 75.3% viability of keratinocytes was observed, versus 53.9% in 0.1 mM Ca^{2+} HK medium, after the cells were harvested by trypsin and EDTA. Thus, not only

Table I. Number of Keratinocytes Grown in HK Medium Containing Sr^{2+}[a]

Sr^{2+} (mM)	Cell Number per Well ($\times 10^{-5}$)	
	0.03mM Ca^{2+}	Ca^{2+}-free
3.0	2.25 (0.33)	1.20 (0.35)
2.0	3.20 (0.50)	2.59 (0.60)
1.0	2.57 (0.49)	2.48 (0.57)
0.3	1.43 (0.56)	1.25 (0.38)
0	1.01 (0.41)	0.67 (0.39)

[a] 1×10^5 cells were plated on 35-mm dishes and cultured for 7 days. Cells were harvested in trypsin and EDTA solution and counted with a hemocytometer. Results are the means (± 1 SD) from three experiments.

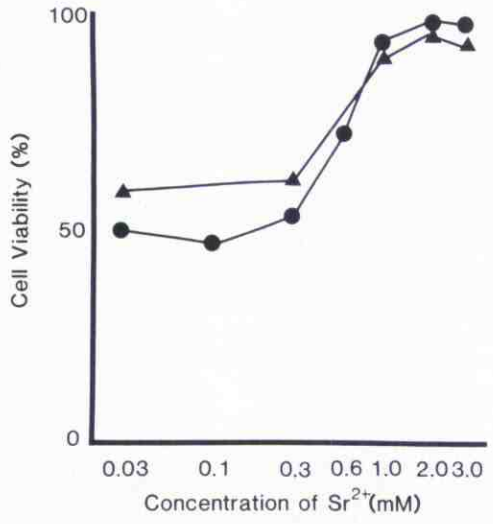

Figure 1. Viability of keratinocytes cultured for 7 days in base HK medium containing Sr^{2+} by AO/EB staining method. Cells were cultured on Lab-Tek chamber slides and viability of attached cells was examined without any trypsin or EDTA treatment. Each point is the mean of three different experiments. In each experiment, at least 500 cells were counted. ●—●, cells in 0.03 mM Ca^{2+} HK medium; ▲—▲, cells in Ca^{2+}-free HK medium.

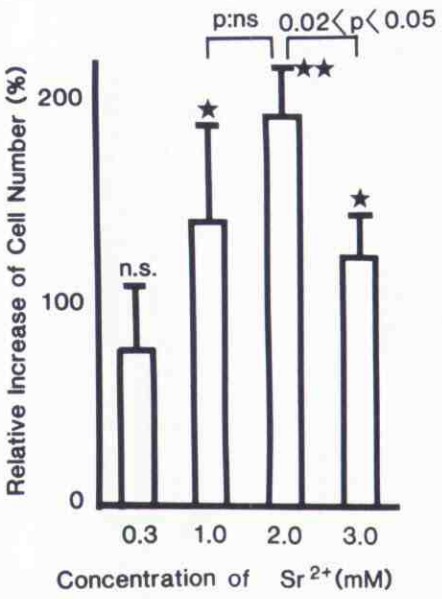

Figure 2. Relative number of keratinocytes grown in base HK medium containing Sr^{2+}. 1×10^5 cells were plated on 35-mm dishes and cultured for 7 days. Cells harvested in trypsin and EDTA solution were counted with a hemocytometer. The relative increase in cells is expressed as a percentage (the number of cells in base HK medium with 0.1 mM Ca^{2+} was estimated to be 100%). Each point is the mean of three separate experiments. Compared with base HK medium, 1.0–2.0 mM Sr^{2+} showed a significant increase in cell number (*$0.02 < p < 0.05$, **$0.001 < p. < 0.01$). A significant difference was found between 2.0 and 3.0 mM Sr^{2+} medium ($0.02 < p < 0.05$), but not between 1.0 and 2.0 mM Sr^{2+} medium. n.s., not significant.

Table II. Viability of Cultured Keratinocytes Harvested in Trypsin and EDTA Solution[a]

Experiment	Viability (%)	
	Base HK Medium + 2.0 mM Sr^{2+}[b]	Base HK Medium + 0.1 mM Ca^{2+}
1	84.0	43.0
2	72.9	65.6
3	91.3	70.0
4	61.9	58.3
5	66.6	32.5
Mean	75.3 (10.9)[c,*]	53.9 (14.1)

[a] Five separate keratinocyte cultures were maintained for 7 days in 60-mm dishes containing base HK medium with Sr^{2+} or Ca^{2+}. Cultured cells were harvested in 0.05% trypsin and 0.01% EDTA solution for 5 min at 37°C and for 5 min at room temperature. Cell viability was determined by the trypan blue dye exclusion test.
[b] 2.0 mM Sr^{2+} HK medium contains base concentration of 0.03 mM Ca^{2+}.
[c] The number in parentheses is one standard deviation.
* Significant increase, $0.02 < p < 0.05$ (versus mean viability of cells in Ca^{2+} medium).

is the viability of cells rather higher with Sr^{2+} (Fig 1), but the viability of cells placed in suspension by trypsinization is higher after growth in Sr^{2+}.

Effects of Sr^{2+} on Growth of Skin Cells In preliminary experiments, we found that 0.1 mM Ca^{2+} produced the best numerical keratinocyte growth of the Ca^{2+} concentrations studied, and we used this concentration in subsequent comparisons with Sr^{2+} in 0.03 mM Ca^{2+}.

Figure 3 shows the time course of the number of cultured keratinocytes in the base HK medium containing 2.0 mM Sr^{2+} (plus 0.03 mM Ca^{2+}) or in medium containing 0.1 mM Ca^{2+}. On days 1 and 3, cell number in each medium was similar. By day 7, cell number in base HK medium with 2.0 mM Sr^{2+} was significantly increased compared with that in base HK medium with 0.1 mM Ca^{2+}.

In contrast, 2.0 mM Sr^{2+} had no effects on growth of cultured melanocytes, and 2.0 mM Sr^{2+} enhanced fibroblast attachment and

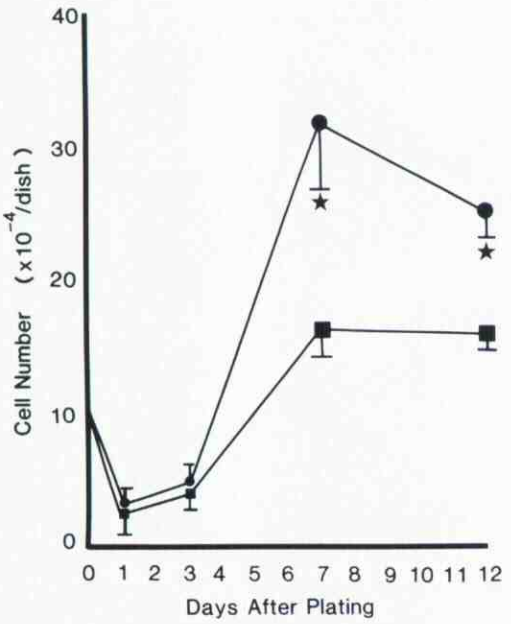

Figure 3. Growth of cultured keratinocytes in base HK medium containing 2.0 mM Sr^{2+} or 0.1 mM Ca^{2+}. 1×10^5 second-passage cells were plated on 35-mm dishes and, after various numbers of days, were harvested in trypsin and EDTA solution and counted. Each point is the mean (±1 SD) from three separate experiments. ●—●, cells in 2.0 mM Sr^{2+} HK medium; ■—■, cells in 0.1 mM Ca^{2+} HK medium. *$0.001 < p < 0.01$.

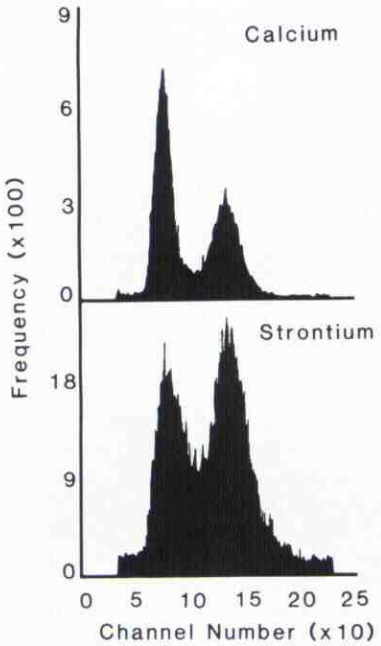

Figure 4. Histogram of cell cycle analysis of cultured keratinocytes in base HK medium containing 2.0 mM Sr^{2+} or 0.1 mM Ca^{2+}. These results are for cells cultured 10 days on 60-mm dishes.

enhanced subsequent cell number proportionally to the increased initial cell attachment (data not shown).

Effects of Sr^{2+} on the Cell Cycle Marked changes of cell cycle pattern were demonstrated for keratinocytes cultured in base HK medium containing 2.0 mM Sr^{2+}. Figure 4 shows representative comparative histograms of the cell cycle of cultured keratinocytes in 2.0 mM Sr^{2+} HK medium and 0.1 mM Ca^{2+} HK medium. The percentage of G_2/M-phase keratinocytes increased significantly from 38 to 55% when cells were cultured in 2.0 mM Sr^{2+}. In contrast, the percentage of cells in G_0/G_1 phase decreased from 54 to 33%. Table III shows the change of cell cycle pattern of keratinocytes cultured in 2.0 mM Sr^{2+} HK medium and 0.1 mM Ca^{2+} HK medium based on three different experiments, and again demonstrates a significant increase of G_2/M phase and decrease of G_0/G_1 phase in keratinocytes cultured in Sr^{2+} HK medium.

The unchanged percentage of cells in S phase confirms the [^{3}H]thymidine incorporation studies that showed a 1.6-fold increase in [^{3}H]thymidine incorporation of cultured cells between 2.0 mM Sr^{2+} medium and 0.1 mM Ca^{2+} medium (Table IV). This increase represents the quantitative increase in cell number. Meanwhile, melanocytes or fibroblasts cultured in 2.0 mM Sr^{2+} HK medium showed no significant cell cycle changes.

Effects of Sr^{2+} on Keratinocyte Density Addition of 2.0 mM Sr^{2+} produced an increase from 56.4 to 79.3% (mean of three experiments) of low-density keratinocytes detected within the 1.017–

Table III. Effects of Sr^{2+} on Cell Cycle of Keratinocytes[a]

Cell Cycle	Base HK Medium + 2.0 mM Sr^{2+}[b]	Base HK Medium + 0.1 mM Ca^{2+}
G_0/G_1	36.1 (3.3)	53.3 (4.7)*
S	14.1 (2.6)	11.9 (2.5)
G_2/M	49.8 (5.9)	34.7 (4.5)**

[a] Results are the mean percentages (±1 SD) of three different experiments.
[b] Sr^{2+} HK medium contains 0.03 mM Ca^{2+}.
* $0.001 < p < 0.01$.
** $0.02 < p < 0.05$.

Table IV. [^{3}H]Thymidine Uptake of Keratinocytes Cultured in Sr^{2+} Medium

HK medium	[^{3}H]Thymidine Uptake[a]	
	Day 4	Day 6
2.0 mM Sr^{2+} + 0.03 mM Ca^{2+}	2125.0 (148.6)	2887.1 (426.9)
0.1 mM Ca^{2+}	1380.7 (414.8)	1976.3 (607.4)
0.03 mM Ca^{2+}	448.7 (185.7)	581.4 (203.1)

[a] Results are the mean cpm (±1 SD) of three experiments. Initially, 5×10^4 cells were plated in each well.

1.033 range of a continuous density gradient generated from 30% Percoll in Hanks' balanced salt solution.

Immunofluorescence Staining Pattern There were no significant changes in the staining patterns of keratinocytes stained with anti-human keratin antibodies, MoAb to skin basal cells, and sera from the patients with pemphigus vulgaris and bullous pemphigoid. We found a striking difference in the incidence of positive cells stained with anti-HFN. Positive staining was found in 81.2% (±9.1) of keratinocytes in 2.0 mM Sr^{2+} HK medium compared with 51.9% (9.5) in 0.1 mM Ca^{2+} HK medium ($0.001 < p < 0.01$). Strong positive staining in cells in Sr^{2+} medium was demonstrated in the cytoplasm with little intensity of extracellular HFN (Fig 5*a*). In contrast, cells in Ca^{2+} 0.1 mM HK medium had cytoplasmic and extracellular positive staining of HFN (Fig 5*b*). Flow cytometric analysis of cell surface HFN confirmed the significant increase in positive staining cells in Sr^{2+} containing-medium (Fig 6).

For melanocytes or fibroblasts cultured in Sr^{2+} and Ca^{2+} medium, there were no differences in the staining pattern to anti-vimentin, anti-HFN, anti-S100, and anti-basal cell antibodies.

DISCUSSION

Sr^{2+}, a divalent cation, substitutes for Ca^{2+} in stimulating histamine secretion from mast cells [14], in stimulating locomotion of epidermal cells from *Xenopus laevis* [33], as a cofactor in human skin collagenase activity [34], and in stimulating methacholine-evoked sweat secretion [15]. Unlike Ca^{2+} however, Sr^{2+} has little effect on fibroblast spreading [35] or on cell adhesion of mouse lymphoblastoma cells [36]. There have been few reports on the effects of Sr^{2+} on cultured skin cells. Most recently, Praeger et al [16] examined the Sr^{2+} effects on keratinocytes cultured in M199–$CaCl_2$ (0.03 mM Ca^{2+}) medium and showed that addition of Sr^{2+} could almost double the number of keratinocytes that grew in medium containing Ca^{2+} as the major divalent cation. They showed that optimal additional Sr^{2+} doubled the number of cultured keratinocytes with no stratification and suggested that Sr^{2+} was a potent mitogen for human keratinocytes. The replacement of Ca^{2+} by Sr^{2+} is currently incompletely studied, however. Therefore, the present study was designed to better characterize the effects of Sr^{2+} on cultured skin cells and to verify whether or not cultured keratinocytes stimulated with Sr^{2+} provide new insights into the biology of cultured keratinocytes.

The initial studies showed that Sr^{2+} (1.0–2.0 mM) increased the number of viable cells in Ca^{2+}-free medium to a level similar to that of cells cultured in base HK (0.03 mM Ca^{2+}) medium, which indicated that Sr^{2+} could substitute for Ca^{2+} in increasing all growth. However, high levels of Sr^{2+} did not induce stratification, as is seen in the high Ca^{2+} concentration. In addition, viability of cells on Lab-Tek chambers with added Sr^{2+} in either 0.03 mM Ca^{2+} or Ca^{2+}-free HK medium showed a similar dose-dependent increase. At lower concentrations of Sr^{2+} (0.03–0.6 mM), 40–50% of cells in chamber wells were not viable. This might be due to high sensitivity of the AO/EB staining method or decreased Ca^{2+} concentration of the HK medium used in this test. We believe this probably reflects the tenuous state of cells grown in primary and first-passage culture in 0.1 mM Ca^{2+} and then subcultured to low Ca^{2+}, low Sr^{2+} medium.

Cultured keratinocytes stimulated with Sr^{2+} at 1.0–3.0 mM showed higher viability and an almost twofold increase in cell number, although lower concentrations of Sr^{2+} did not show beneficial effects. These results are compatible with the report of Praeger et al [16]. On the basis of our experiments, 2.0 mM Sr^{2+} is optimal for keratinocyte cultures. Although 2.0 mM Sr^{2+} had no effects on the cell cycle and growth of cultured human skin melanocytes or fibro-

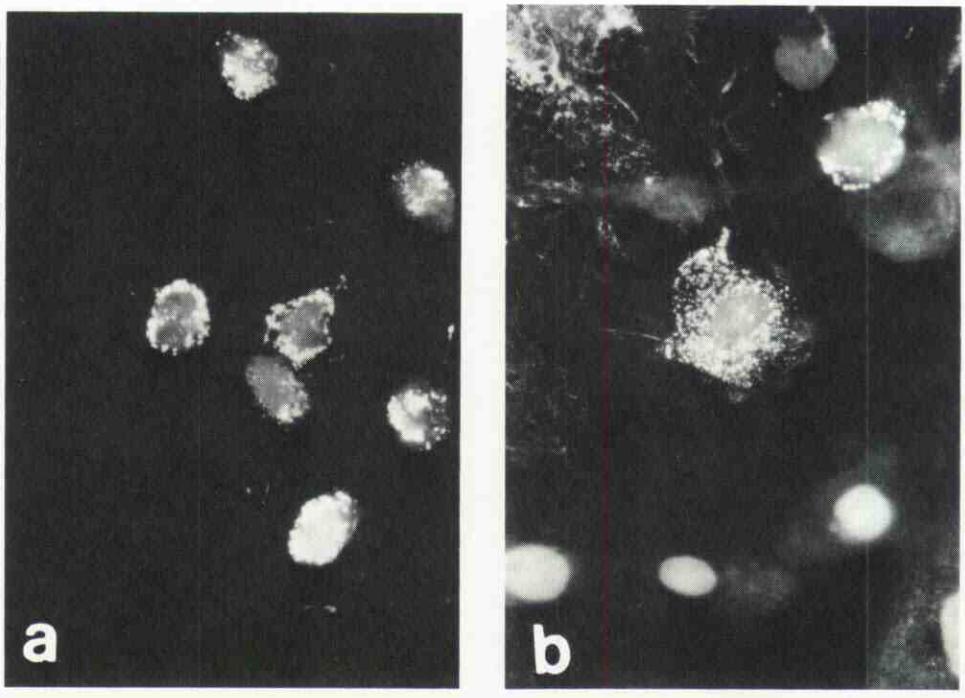

Figure 5. Fluorescent photomicrographs of cultured keratinocytes stained with FITC-conjugated anti-human fibronectin antibody. *(a)* Cells cultured in 2.0 mM Sr^{2+} HK medium. *(b)* Cells cultured in 0.1 mM Ca^{2+} HK medium. Cells were grown on Lab-Tek chamber slides after 6 days in medium and prefixed by acetone. The nucleus was counterstained with PI.

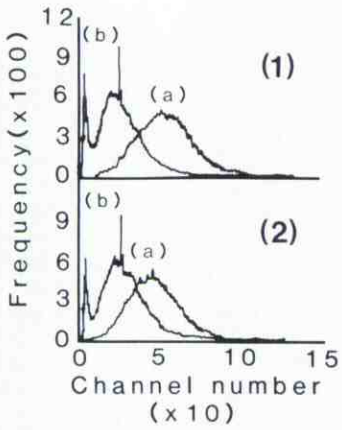

Figure 6. Flow cytometry analysis of cultured keratinocytes stained with FITC-labeled anti-human fibronectin antisera. *(1)* Keratinocytes cultured in Sr^{2+} HK medium. *(2)* Keratinocytes cultured in Ca^{2+} HK medium. *(a)* FITC-labeled anti-HFN antiserum staining pattern. *(b)* FITC-labeled anti-human IgG staining pattern as control. Positive ratio is 65.0% in histogram 1 and 49.27% in histogram 2.

blasts, the possibility remains that the optimal concentration of Sr^{2+} for cultured melanocytes or fibroblasts is different from that for cultured keratinocytes.

The effect of Sr^{2+} on keratinocyte cell cycle was to increase the percentage of cells in the S and G_2/M phases with a coincident decrease in cells in G_0/G_1, probably reflecting a decrease in cells in G_0. This produced a more uniform population of proliferative, but not differentiating, keratinocytes. Keratinocytes stimulated with Sr^{2+} showed a significant increase in the number of healthy cuboidal cultured cells in G_2/M phase and seemed to be resistant to the damage by the trypsin treatment required for cell harvest. Similar effects on proliferation and cell cycle were not seen with cultured melanocytes or fibroblasts.

Fibronectin is an important matrix protein involved in cell attachment and movement. Several studies of fibronectin during re-epithelialization of cutaneous wounds [37,38] and in basal cell epithelioma [39,40] proposed that epidermal keratinocytes produced fibronectin. These reports suggest that the production of HFN is augmented in the reactive or neoplastic proliferation of basal cells. Several studies have convincingly demonstrated that human keratinocytes synthesize, secrete, and deposit fibronectin in the extracellular matrix in culture, and perhaps in intracellular locations in the epidermis [41–43]. We have shown that strontium increases the population of cultured keratinocytes containing HFN in the cytoplasm and on the cell membrane. As it is clear that the basal keratinocyte population expands during cell culture [6,44], the enhanced expression of HFN by strontium may enhance cell–matrix interactions and cell movement.

All cell culture media contain large amounts of various divalent cations. Therefore, it is difficult to determine the real role of Sr^{2+} on cultured keratinocytes and to rule out the interaction between Sr^{2+} and other divalent cations on cultured keratinocytes. With the present system of keratinocyte culture with the medium containing minimal Ca^{2+} concentration (0.03 mM), however, additive Sr^{2+} induced some significant changes in cell growth, cell viability, cell cycle, and HFN staining pattern. Even in totally Ca^{2+}-free medium, Sr^{2+} effectively augmented keratinocyte growth. Based on the report of Praeger et al [16] and our present results, it can be concluded that Sr^{2+} plays a characteristic role in keratinocyte proliferation and differentiation in serum-free conditions in vitro. The effect of Sr^{2+} in these cultures is not simply to substitute for Ca^{2+}. Whereas elevation of Ca^{2+} induces keratinocyte differentiation and stratification, Sr^{2+} induces dedifferentiation and the production of a more uniform proliferative basaloid cell population.

These results show that Sr^{2+} can be of practical benefit in the culture of human keratinocytes in serum-free medium, increasing the viability and proliferative rate, and producing a more uniform population of basaloid cells with increased expression of cytoplasmic and cell surface fibronectin.

We thank Professor Sadao Imamura, Kyoto University, Japan, for providing monoclonal antibody to skin basal cell.

REFERENCES

1. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell 6:331–344, 1975
2. Liu SC, Karasek M: Isolation and growth of adult human epidermal keratinocytes in cell culture. J Invest Dermatol 71:157–162, 1978
3. Gilchrest BA, Calhoun JK, Maciag T: Attachment and growth of human keratinocytes in a serum-free environment. J Cell Physiol 112:197–206, 1982
4. Gilchrest BA, Nemore RE, Maciag T: Growth of human keratinocytes on fibronectin-coated plates. Cell Biol Int Rep 11:1009–1016, 1980
5. Karasek MA, Charlton NE: Growth of post embryonic skin epithelial cells on collagen gels. J Invest Dermatol 56:205–210, 1971
6. Furukawa F, Huff JC, Weston WL, Norris DA: Serum-free serial culture of adult human keratinocytes from suction-blister roof epidermis. J Invest Dermatol 89:460–463, 1987
7. Rheinwald JG, Green H: Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Science 265:421–424, 1977
8. Hayashi I, Larner J, Sato G: Hormonal growth control of cells in culture. In Vitro 14:23–30, 1978
9. Maciag T, Nemore RE, Weinstein R, Gilchrest BA: An endocrine approach to the control of epidermal growth: Serum-free cultivation of human keratinocytes. Science 211:1452–1454, 1981
10. Eisinger M: Cultivation of normal human epidermal keratinocytes and melanocytes. In: Methods in Skin Research. Edited by D. Skerrow and CJ Skerrow. John Wiley & Sons, New York, 1985, pp 191–212
11. Ham RG: Nutritional requirement of primary cultures. A neglected problem of modern biology. In Vitro 10:119–129, 1974
12. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R: An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci USA 76:5674–5678, 1979
13. Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum free serial culture. J Invest Dermatol 81:33s–40s, 1983
14. Foreman JC: Spontaneous histamine secretion from mast cells in the presence of strontium. J Physiol 271:215–232, 1977
15. Sato K, Sato F: Spontaneous sweat secretion in calcium-free strontium medium. Am J Physiol 242:C353–C359, 1982
16. Praeger FC, Stanulis-Praeger BM, Gilchrest BA: Use of strontium to separate calcium-dependent pathways for proliferation and differentiation in human keratinocytes. J Cell Physiol 132:81–89, 1987
17. Mishell BB, Shiigi SM: *In vitro* immune responses. In: Selected Methods in Cellular Immunology. Edited by BB Mishell and SM Shiigi. Freeman, San Francisco, 1980, pp 1–172
18. Parks DR, Bryan VM, Oi VT, Herzenberg LA: Antigen specific identification and cloning of hybridomas with a fluorescence activated cell sorter. Proc Natl Acad Sci USA 76:1962–1965, 1979
19. Crissman HA, Steinkamp JA: Rapid simultaneous measurement of DNA protein and cell volume in single cells from large mammalian cell populations. J Cell Biol 59:766–771, 1973
20. Crissman HA, Egmond JV, Holdrinet RS, Pennings A, Haanen C: Simplified method for DNA and protein staining of human hematopoietic cell samples. Cytometry 2:59–62, 1981
21. Krishan A: Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66:188–193, 1975
22. Dean PN, Jett JH: Mathematical analysis of DNA distributions derived from microfluorometry. J Cell Biol 60:523–527, 1974
23. Gray JW: Cell cycle analysis from computer synthesis of deoxyribonucleic acid histogram. J Histochem Cytochem 22:642–650, 1974

24. Fischer SM, Nelson KD, Reiners JJ, Viaje A, Pelling JC, Slaga TJ: Separation of epidermal cells by density centrifugation: A new technique for studies on normal and pathological differentiation. J Cutan Pathol 9:43–49, 1982
25. Simon M, Green H: Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell 36:827–834, 1984
26. Smith EL, Walworth NC, Holick MF: Effect of α,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J Invest Dermatol 86:709–714, 1986
27. Grimwood RE, Huff JC, Weston WL: A simple and improved method for direct and indirect immunofluorescent staining. J Am Acad Dermatol 13:768–771, 1985
28. Ockleford CD, Hsi BL, Wakely J, Badley RA, Whyte A, Faulk WP: Propidium iodide as a nuclear marker in immunofluorescence. 1. Use with tissue and cytoskeleton studies. J Immunol Methods 43:261–267, 1981
29. Huff JC, Weston WL, Wanda KD: Enhancement of specific immunofluorescent findings with use of a para-phenylenediamine mounting buffer. J Invest Dermatol 78:449–450, 1982
30. Oh SH, Furukawa F, Kashihara M, Horiguchi Y, Imamura S: Clinical application of monoclonal antibody to basal cell of the human skin. Acta Dermatol (Kyoto) 81:307–314, 1986
31. Lanier LL, Warner NL: Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J Immunol Methods 47:25–30, 1981
32. Haftek M, Viac J, Cordier G, Thivolet J: Flow cytometry for separation of keratinocyte subpopulations from the viable epidermis. J Invest Dermatol 87:480–484, 1986
33. Strohmeier R, Bereiter-Hahn J: Control of cell shape and locomotion by external calcium. Exp Cell Res 154:412–420, 1984
34. Seltzer JL, Welgus HG, Jeffrey JJ, Eisen AZ: The function of Ca^{2+} in the action of mammalian collagenases. Arch Biochem Biophys 173:355–361, 1976
35. Yasuda K: Studies on the factors affecting cellular spreading upon culture-substrate. 1. The effects of divalent cations and conditioned medium on cellular spreading. J Cell Sci 15:269–278, 1974
36. Attramadal A: The effect of divalent cations on cell adhesion. J Periodont Res 4:281–285, 1969
37. Fujikawa LS, Foster CS, Harrist JJ, Lanigan JM, Colvin RB: Fibronectin in healing rabbit corneal wounds. Lab Invest 45:120–129, 1981
38. Clark RAF, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. J Invest Dermatol 79:264–269, 1982
39. Nelson DL, Little CD, Balian G: Distribution of fibronectin and laminin in basal cell epithelioma. J Invest Dermatol 80:446–452, 1983
40. Grimwood RE, Huff JC, Harbell JW, Clark RAF: The source of fibronectin in basal cell epithelioma. J Invest Dermatol 82:145–149, 1984
41. Kariniemi A-L, Lehto V-P, Varito T, Virtanen I: Cytoskeleton and pericellular matrix organization of pure adult human keratinocytes cultures from suction-blister roof epidermis. J Cell Sci 58:49–61, 1982
42. O'Keefe EJ, Woodley D, Castillo G, Russell N, Payne RE Jr: Production of soluble and cell-associated fibronectin by cultured keratinocytes. J Invest Dermatol 82:150–155, 1984
43. Kubo M, Norris DA, Howell SE, Ryan SR, Clark RAF: Human keratinocytes synthesize, secrete and deposit fibronectin in the pericellular matrix. J Invest Dermatol 82:580–586, 1984
44. Toda KI, Grinnell F: Activation of human keratinocyte fibronectin receptor function in relation to other ligand–receptor interactions. J Invest Dermatol 88:412–417, 1987